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It is characteristic of Science and Progress that they continually open new fields to our vision.—PASTEUR.

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STRAIN VARIATIONS IN C. BIFERMENTANS[1]

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Clostridium bifermentans was first isolated and described by Tissier and Martelly (1902), and was generally accepted as a distinct and definite species until Hall (1922) observed that some bifermentans strains were motile, therein differing from the original description. Correlated with this difference in motility, he found certain other differences—in agglutination and in deep colony morphology. He proposed dividing this species into two species, retaining the name *C. bifermentans* for the non-motile strains, and designating the motile strains as *C. centrosporogenes*.

Kahn (1924), after an extensive comparative study of the anaerobes, recognized this designation, but suggested that perhaps it should be regarded as "a strain variant of *C. sporogenes* and that the cultural features exhibited by Hall's type are not sufficient to warrant a new specific name." Similarly Weinberg and Ginsbourg (1927), in a review of proposed new species, state that *C. centrosporogenes* is merely a particular strain (souche particulière) of *C. sporogenes*.

Sturges and Drake (1928), however, examining Hall's strains of *C. bifermentans* and *C. centrosporogenes* in sealed capillary tubes, found motility in all strains examined, and suggested that *C. centrosporogenes* was probably much more closely related to *C. bifermentans* than to *C. sporogenes*.

The proteolytic ability of *C. bifermentans* is another point on which the results of different investigators vary. Tissier and Martelly (1902), Hall (1922), Reddish and Rettger (1924) and

[1] Presented at the Thirtieth Annual Meeting of the Society of American Bacteriologists, Richmond, Va., December 27, 1928.

Weinberg and Ginsbourg (1927) considered this species as strongly proteolytic. Kendall, Day, and Walker (1922) found moderate proteolysis, and Kahn (1924) reported that proteolysis was "definitely mild in type."

This reported variance in the proteolysis of *C. bifermentans* (which name in some cases undoubtedly included *C. centrosporogenes* since some of the results were reported before Hall's proposed division of the species) was readily accounted for when a preliminary investigation was made of the proteolytic ability of one of Hall's bifermentans and one of his centrosporogenes strains. The comparatively slow action of the bifermentans strain suggested that Hall's division of the group might be substantiated by another and a more definite criterion than aerobic motility tests.

It was further planned to study fermentation reactions, morphology, colony form, and other possible indications of a logical grouping. To show the similarities and dissimilarities of *C. sporogenes* to the *C. bifermentans* group, the former organism was included in all tests.

EXPERIMENTAL

Strains used

The strains used in this study were:

1. *C. bifermentans**.....Lister Institute
2. *C. bifermentans*.....Hall 50
3. *C. bifermentans*.....Hall 70
4. *C. bifermentans*.....Hall 258
5. *C. bifermentans*.....Hall 408 C
6. *C. centrosporogenes*.....Hall 76
7. *C. centrosporogenes*.....Hall 262
8. *C. centrosporogenes*.....Hall 472
9. *C. centrosporogenes*.....Hall 516

10 to 21 inclusive. Strains isolated in this laboratory:

C. sporogenes.....Hall 52
C. sporogenes.....Hall 54

* It should be noted that this strain was named before a division into bifermentans and centrosporogenes had been suggested.

Purity of these strains was insured by plating and by repeated examinations. In the experimental work, every culture was examined for contamination.

Morphology

The most consistent and convenient observations on morphology were made on a minced pork medium, buffered to pH 8.0 with sodium phosphate. Cultures were grown at 37°C., and Gram stains made. Measurements are given for a typical strain (no. 1) in table 1. The reason for a departure from the usual

TABLE 1

Measurements of strain 1

	WIDTH			LENGTH		
	Minimum	Majority	Maximum	Minimum	Majority	Maximum
	μ	μ	μ	μ	μ	μ
Vegetative rods:						
16 hours	0.5	0.7	1.1	1.3	2.5	7.0
40 hours	0.5	0.9	1.6	1.0	4.0	6.5
3 days	1.2	1.4	1.6	2.2	4.5	6.0
7 days	0.9	1.0	1.1	4.0	8.0	260.0
Sporangia:						
All ages	0.7	0.8	1.1	1.8	2.5	3.5
Free spores:						
All ages	0.6	0.8	0.9	1.0	1.4	2.2

custom of reporting measurements in a few figures is obvious. Almost all rods and sporangia are Gram-positive, regardless of age. Spores swell the rods slightly, but definitely, in most of the sporangia. Apparently about 95 per cent of the cells form spores.

While most strains rarely show more than three or four rods in a chain, strains 9, 12, 13 and 14 often have as many as twenty in a chain. In strains 9 and 14, these chains break up as the sporangia develop, but in strains 12 and 13 long chains of mature sporangia are seen. Strains 2, 3, 5, and 7 show little, if any, growth before twenty hours. Vegetative rods measure about the same as strain 1, but sporangia are larger, measuring 0.9 to 1.8μ by 2.5 to 5.0μ. Sporangia of strains 12 and 13 are inter-

mediate in width, this measurement varying from 1.2 to 1.4μ. Strain 8 is exceptional in that the spores rarely swell the rod.

C. sporogenes, under the same conditions, measures 0.7 to 0.9μ by 3 to 5μ at sixteen hours, and 0.5 to 0.7μ by 1 to 4μ at forty hours. After forty hours most of the cells become Gram-negative. Less than 25 per cent of the cells form spores. The spores swell the rods markedly.

Motility was demonstrated for all these 21 strains by examination in sealed capillary tubes. Examined under cover slips, young cultures of strains 1, 5, 6, and 8 to 21 usually, but not always, showed a few motile cells. Motility was but rarely demonstrated by this method for strains 2, 3, 4, and 7. *C. sporogenes* cultures usually contain a large number of motile cells, regardless of the method of examination or age of culture.

Colony form

Thompson (1926) claimed to be able to differentiate between *C. bifermentans* and *C. centrosporogenes* by the types of colonies formed on blood agar. Accordingly, dilution plates were poured, using a veal infusion agar (pH 7.5). To each plate, 0.5 cc. of fresh sterile defibrinated beef blood was added before the agar was poured. These plates were incubated in an atmosphere of hydrogen at 37°C. for forty-eight hours.

Under these conditions, deep colonies develop to a diameter of from 0.1 to 1.0 mm. Several types of colonies may be observed; ameboid masses, containing irregular granules of varying size and density; colonies of simple lens shape; dense lobate colonies appearing to be aggregates of 5 to 20 lens-shaped lobes; and irregular masses with small bud-like protuberances. Crowded plates show only the first type of colony. Edges of colonies may be either entire or irregular to filamentous.

A zone of hemolysis surrounds each colony. Strains 2, 3, 4 and 7 produce a narrow zone of hemolysis, while the other strains produce a comparatively wide zone—as wide or wider than the diameter of the colony. The hemolytic zone of *C. sporogenes* is several times as wide as the diameter of its colony.

Viscosity

Kahn (1924) mentioned a "mucoid-like deposit" in cultures of *C. centrosporogenes*. We have made similar observations in bouillon, gelatin, and meat media. Strain 1, alone, consistently shows this property. In general 2, 3, 4, 7, 12 and 13 usually show a minimum of ropiness or none at all, while the other strains usually show a considerable amount. *C. sporogenes* never produces any viscosity.

Correlated with this property of viscosity is the occurrence of a clear zone in the upper part of gelatin and broth cultures.

TABLE 2

Acid production from carbohydrates

	GLUCOSE	LEVULOSE	GALACTOSE	MANNOSE	MALTOSE	GLYCEROL	SORBITOL
Strains 1, 5, 8 to 12 inclusive..	+	+	0	+	+	+	+
Strains 2, 3, 4, 6 and 7.....	+	+	0	+	+	+	0
C. sporogenes 52 and 54.....	+	+	Slight	0	+	Slight	+

Fermentations

A basic medium containing 1 per cent peptone and 0.3 per cent beef extract was used. To this was added 1 per cent of the carbohydrate. The cultures were incubated three days in hydrogen at 37°C. The pH of the medium before inoculation was between 7.0 and 7.5. Inoculations were made from cultures grown for twenty-four hours in the basic medium. The carbohydrates fermented are shown in table 2. Gas production is variable in the bifermentans group except that it is almost always produced from glucose, maltose and mannose.

Protein metabolism

Observations of the digestive action of these strains on gelatin, coagulated egg white and coagulated blood serum media showed slow liquefaction of these substrates by strains 2, 3, 4 and 7. Digestion by the other strains was more rapid and more extensive. *C. sporogenes* liquefied these media rapidly after a short lag period.

Gelatin was selected as the most suitable medium for obtaining quantitative data for possible differentiation. Progress of proteolysis was measured in terms of electrical conductivity change in the medium. The relation between the production of ammonia, which is an end product of protein metabolism, and conductivity change has been discussed and a technique for the purpose described by Parsons and Sturges (1926).

TABLE 3

Conductivity change in gelatin

Expressed as reciprocal ohms $\times 10^4$

STRAIN	20 HOURS	44 HOURS	4 DAYS	11 DAYS	51 DAYS
1	3.2	7.0	11.7	14.9	18.3
2	1.1	2.1	4.1	9.2	14.9
3	0.4	1.1	3.1	7.3	13.3
4	0.5	1.5	3.7	7.5	11.0
5	3.4	7.9	11.5	15.2	19.4
6	2.5	5.3	10.6	16.8	20.1
7	1.2	3.1	5.4	9.8	12.2
8	2.3	6.3	10.6	14.0	18.8
9	2.2	5.1	8.8	13.9	21.4
10	3.3	5.5	7.4	12.0	18.4
11	2.6	6.6	11.1	15.1	19.4
12	1.9	3.6	5.5	8.9	18.5
13	1.9	3.5	5.3	8.6	20.0
14	2.2	4.4	7.9	13.1	22.3
15	2.5	4.5	8.2	15.1	19.6
16	2.0	4.4	8.1	12.9	18.7
17	1.8	3.5	6.4	12.0	20.4
18	1.8	3.6	6.6	10.6	20.8
19	2.0	5.2	10.1	13.7	20.4
20	3.8	8.9	14.4	16.8	21.8
21	2.9	5.6	10.2	13.4	17.3
C. sporogenes 52	0.2	13.4	20.0	24.4	28.0
C. sporogenes 54	0.5	12.2	18.6	21.9	26.2

Nutrient gelatin was inoculated from twenty-four-hour cultures of each strain, and incubated in an atmosphere of hydrogen at 37°C. Resistances of cultures were read at twenty hours, forty-four hours, four days, eleven days, and fifty-one days.

Ten tubes were inoculated with each strain, and duplicate tubes were used at each interval. Readings were made at 30°C.

From the resistance, conductivity was computed, and the similarly determined conductivity of the uninoculated controls was subtracted. Conductivity change is expressed in reciprocal ohms $\times 10^3$. For the sake of brevity and clarity, and since the readings on duplicate tubes showed but few deviations of more than 2 per cent from the average, only the averages are reported in table 3.

DISCUSSION

The data on morphology show several points of difference between *C. sporogenes* and all the other strains. The former is more persistent in its motility; its spores swell the rods more; vegetative rods of *C. sporogenes* cultures become smaller as the cultures mature, while rods in cultures of the bifermentans group become larger; the majority of the rods in *C. sporogenes* cultures lose their property of retaining the Gram stain, decrease in size and die without sporulating, while most of the organisms in cultures of the bifermentans group produce spores.

Several possible groupings of the 21 strains are suggested by the data. Number 8 differs in the fact that its spore does not swell the rod. Strains 9, 12, 13 and 14 show more chain formation, 12 and 13 being more persistent in this respect. Strains 2, 3, 4 and 7 differ in the slowness of their growth, the sensitiveness of their motility, and the width of their sporangia. Strains 12 and 13 are intermediate with regard to sporangium width.

As to colony formation, no consistent differences are observed, except in the ratio of the diameter of the colony to the width of the zone of hemolysis. *C. sporogenes* is distinctly more hemolytic. Strains 2, 3, 4 and 7 are feebly hemolytic. The other strains are intermediate.

The production of viscosity again distinguishes *C. sporogenes* from the bifermentans group. It also gives an indication, although an undependable one, of a differentiation between strains 2, 3, 4, 7, 12 and 13, and the remainder of the group.

The fermentation of mannose furnishes a definite and precise

differentiation of *C. sporogenes* from the bifermentans group. The reactions in galactose and glycerol, although less definite, appear to be constant. The fermentation of sorbitol demarcates strains 2, 3, 4, 6 and 7 from the remainder of this group.

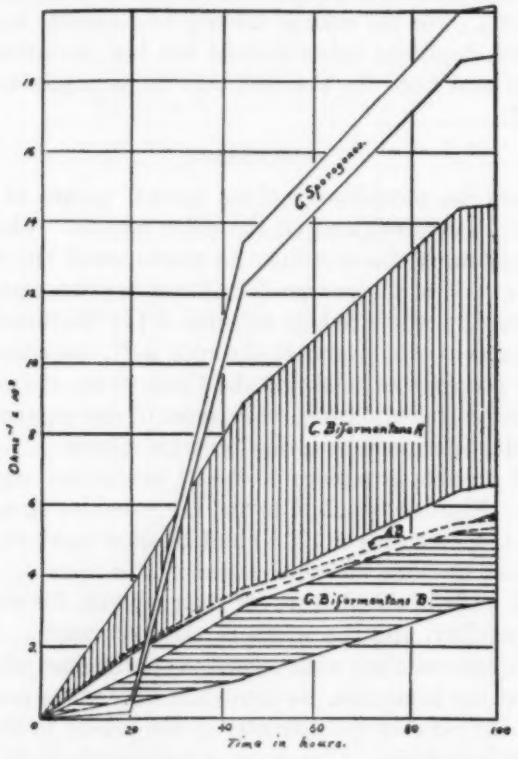

FIG. 1. RANGE OF CONDUCTIVITY CHANGE IN GELATIN, ALL STRAINS—EARLY AGES

The data on digestion of gelatin in table 3 suggest no basis for a division of the bifermentans strains. However, in figures 1 and 2 we have divided these strains into groups on the basis of previously described differences in morphology, hemolysis, viscosity and macroscopic observations of liquefaction of proteins.

The highest and the lowest values (conductivity change) for each day in each group are plotted as points. The areas between the lines connecting these points will be referred to as zones. The zoning arrived at in this manner gives some support to these other differentiations as shown by the separation of the zone designated *A* from that designated *B*.

Strains 12 and 13, however, exhibit an intermediate type of activity as was the case with their other properties. The course

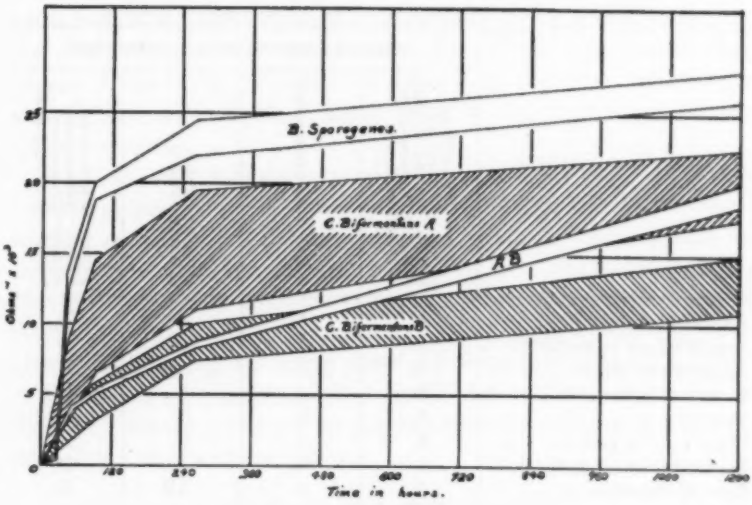

FIG. 2. RANGE OF CONDUCTIVITY CHANGE—OLD AGES

of their gelatin digestion is represented by the narrow zone, *AB*, which for the first forty-five hours lies within zone *A*, then deflects to enter zone *B* at about ninety hours, emerging again (see fig. 2) and reëntering zone *A*. If, therefore, these two cultures should be included either in Group A or in Group B, the reconstructed zones would show a considerable overlapping and any such differentiation would be impossible. Similarly, a grouping made according to the fermentation of sorbitol would give widely overlapping zones.

The difference between the bifermentans group and *C. sporogenes*, in gelatinolytic activity, is so clearly shown in the charts as scarcely to need comment. The latter shows a lag period extending for twenty hours, followed by a period of maximum activity, during which proteolysis, as measured by ammonia accumulation, proceeds to a point never attained by members of the bifermentans group.

TABLE 4

Grouping of bifermentans strains according to type of reaction

DIFFERENTIAL CHARACTERS	STRAINS, SOURCES AND PREVIOUS DESIGNATION			
	1 (Lister) "Bifermentans" 5 (Hall) "Bifermentans" 8 (Hall) "Centrosporogenes" 9 (Hall) "Centrosporogenes" 10–21 Authors' Strains	6 (Hall) "Centrosporogenes"	12 Authors' Strains 13 Authors' Strains	2 (Hall) "Bifermentans" 3 (Hall) "Bifermentans" 4 (Hall) "Bifermentans" 7 (Hall) "Centrosporogenes"
Rapidity of growth	A	A	A	B
Sensitiveness of motility	A	A	A	B
Sporangium width	A	A	AB	B
Extent of hemolysis	A	A	A	B
Viscosity	A	A	B	B
Clear zone in gelatin	A	A	B	B
Fermentation of sorbitol	A	B	A	B
Rate of digestion	A	A	AB	B

Consideration of these charts will readily show the possibilities of erroneous conclusions when observations of one or two strains are used as a basis for differentiation of anaerobic species. For instance, there are strains of *C. bifermentans* whose position lies nearer to the *C. sporogenes* zone than to the *B* zone.

Another important point illustrated by these charts is the possibility of fallacies arising from observations limited to one or two ages. Thus the conductivity readings for *C. sporogenes* indicate consecutively: first, coincidence with the less proteolytic strains of *C. bifermentans*; then, coincidence with the more pro-

teolytic strains; and finally, a distinct disparity from both. The disagreement as to the extent of proteolysis caused by *C. bifermentans* may possibly be explained in this way.

Each of the characters studied indicates a greater difference between *C. sporogenes* and the bifermentans group than between the individual members of the group. Correlation of the differentiations within the group indicates two types differing in morphology, hemolysis, viscosity, sorbitol fermentation and proteolysis. The reaction common to strains 1, 5, 8, 9, 10, 11, and 14 to 21 inclusive are designated in table 4 as Type A reactions, and the reactions common to strains 2, 3, 4 and 7 as Type B reactions. Strain 6 has Type A reactions except on sorbitol. Strains 12 and 13 have some Type A, some Type B, and some intermediate (AB) reactions.

While the reactions designated as Type A and Type B represent real differences, none of these differences, except sorbitol fermentation, are both sufficiently great and sufficiently constant to be recommended for general classification. Furthermore, the fact that there are cultures giving, in some characters, Type A reactions, and in other characters, Type B or intermediate reactions, militates against using these reactions to divide the species.

Finally, it is to be noted that Hall's differential designation of his eight strains could not be substantiated by differences in any one of the characters studied.

SUMMARY AND CONCLUSIONS

With the assumption that *C. bifermentans* and *C. centrosporogenes* constituted a somewhat unified group, a study was made of all available strains (twenty-one) belonging to this group.

Since no consistent differences were found between the strains labelled *C. bifermentans* and the strains labelled *C. centrosporogenes*, the use of the latter name would seem unjustifiable.

The variations observed suggest another and a more logical grouping, but the nature of the variations and the existence of intermediate strains necessitate all of the strains being considered as a single species.

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USE OF THE MICROSCOPE IN STUDYING THE ACTIVITIES OF BACTERIA IN SOIL[1]

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The microscopic method for studying bacteria in soil was first proposed about twelve years ago (Conn, 1917). It was then employed chiefly as a method for checking up other procedures for counting bacteria in soil and as a means of determining the morphological types of microörganisms present in any given soil. It was felt at the time that its usefulness along these two lines was strictly limited, but it was believed that the future might show other ways in which it could be used to greater advantage. Such has proved to be the case. Winogradsky (1925) has employed the method (with slight changes in technic) for observing the kinds of organisms stimulated by the addition of various ingredients to soil. More recently Rossi (1927) has used a modification of the technic for studying the groupings and colony formation of bacteria in soil while Chudiakov (1926) has employed Winogradsky's technic for studying adsorption of bacteria by soil particles.

The line of investigation suggested by Winogradsky's work has been followed in this laboratory (Thatcher and Conn, 1927) along slightly different lines; and as a result of this study another use of the method has been worked out which promises to be fully as important as those in which it has been previously employed. The use in question is in studying the food requirements of bacteria in soil. The method has been used in this way in certain recent investigations (Conn, 1928a and 1928b) but it appears important enough for greater emphasis than that given in the publications cited.

[1] Paper presented at the meeting of the Society of American Bacteriologists, Richmond, Va., December 28, 1928.

NEED FOR THE METHOD

It has been recognized for some time that the behavior of soil organisms in soil and in laboratory media may be very different. Without question, it is more important to know their behavior in soil, but the difficulty of studying their physiology in such a medium has kept investigators from making much progress in this line. In such a study three serious difficulties have always been encountered. In the first place, pure culture work is impossible unless the soil is sterilized, and sterilization changes its physical and chemical nature. In the second place, even if one assumes that the changes in the soil brought about by sterilization are not sufficient to affect results, one encounters great difficulty in making chemical analysis of the soil; while in the third place, the opacity of the medium renders it impossible to determine by eye whether growth has occurred or not.

The last two difficulties prove decided obstacles, because one cannot study the physiology of an organism unless one can measure its end products, or at least determine whether or not it is actually growing in the medium used for study.

In the method now proposed for the purpose, it must be stated frankly that the first of these difficulties has not been eliminated. No method has yet been found for studying the organisms in soil in pure culture without sterilizing the soil with heat. It is believed, nevertheless, that even after autoclaving, soil is a more natural medium for soil bacteria than are either liquid or agar media. In one or two instances it has been possible to check up the matter, in fact, by comparing the ability of an organism to grow in pure culture in a sterilized soil with its presence or absence on gelatin plates made from the soil in question; and the agreement has been close enough to suggest that heated and unheated soil are not so different as media for the growth of soil bacteria as was originally feared.

The real value of the microscopic method in the physiological study of soil bacteria is that it furnishes a rapid means of determining whether or not an organism is growing in a given soil sample. In other words, it overcomes the handicap of working

with a medium in which the bacterial growth is invisible to the unaided eye.

In employing this method, a soil may be selected in which the organism under investigation does not grow unless special nutrients are added. It may be possible to find a soil so poor in bacterial nutrients that no soil organism will grow in it without such addition. As yet no effort has been made to locate such a soil, but it has proved simple to find one poorly adapted to the growth of the particular organisms thus far investigated. Any nutrient material or mixture of nutrient materials that it is desired to study may be added to this soil; the soil may then be inoculated with the organism under investigation, and its ability to grow may be observed by microscopic examination.

TECHNIC EMPLOYED

The soil is nearly air-dried and passed through a sieve with holes about 2 mm. in diameter. Water is added by spraying until the soil is moist, but still of a good consistency for handling. It is then stored in stoppered bottles and when ready to use 10 grams are placed in each test tube. The best sized test tube is one of 18 to 20 mm. outside diameter. The tubes are plugged with cotton and sterilized thirty minutes at one atmosphere pressure in an autoclave.

The nutrients to be studied are generally added at the rate of 1 gram per 100 grams of soil, but the quantity employed may be varied according to the nature of the ingredient in question. The ingredients may be added in one or the other of two different ways: they may be dissolved in the water sprayed onto the soil before sterilizing; or they may be dissolved in a portion of this water, sterilized separately by filtration and added aseptically to the soil after autoclaving. The latter method is preferable in case of organic compounds subject to possible decomposition on heating.

The cultures used for inoculation are twenty-four-hour-old agar cultures; the growth is rubbed up in sterile distilled water and 1 cc. transferred under aseptic conditions to each tube of sterile soil.

After various periods, generally two days, four days, and six days, samples of 0.5 gram each are removed, with care to avoid contamination, and mixed with 4.5 cc. of a 0.015 per cent solution of gelatin. This gelatin solution is prepared previously and sterilized in test tubes. A drop of this suspension is smeared on a slide, and dried on a flat surface over a boiling water bath. It is then stained by the technic previously described by the writer for the study of bacteria in soil (Conn, 1918) and subsequently modified (Conn, 1928). This later modification is simpler and more controllable than the procedures previously suggested; it is as follows:

Make a suspension of soil in nine times its weight of an 0.015 per cent solution of gelatin. Smear a drop of this in a thin film on a slide. Dry on a flat surface over a boiling water bath. While still on the water bath cover with the following staining fluid:

Rose bengal	1 gram
$CaCl_2$	0.01 gram
5 per cent aqueous phenol	100 cc.

Allow to stand for one minute. Wash as rapidly as possible in tap water; dry and examine under the microscope. A rather high power is necessary, the combination of lenses giving best results of any that have been tried being a 1.9 mm. fluorite objective with a 12.5 compensating or planoscopic ocular.

RESULTS OBTAINED

As an illustration of the use of this method two figures are given. Both of these figures show sterilized soil that was inoculated with an organism recently studied in this laboratory, *Bacterium globiforme*[2] Conn (1928a). Both illustrations are microscopic preparations of the same soil, Volusia silt loam, in which this organism does not ordinarily grow. In the case of figure 1 the soil had no treatment except sterilization. In figure 2 the soil contained 1 gram of ammonium sulfate and 1 gram of glucose per 100 grams of soil. These illustrations suggest that the reason this organism fails to grow in this soil under normal conditions is because the soil

[2] Incorrectly spelled *Bacterium globiformis* when originally described.

lacks the necessary carbon or nitrogen sources, or both. Other investigations already reported (1928a) show that this particular organism requires both carbon and nitrogen added to this soil in order to allow it to grow. These two particular ingredients, however, (ammonium sulfate and glucose) are not the only ones satisfactory for the purpose. The investigation has shown that this

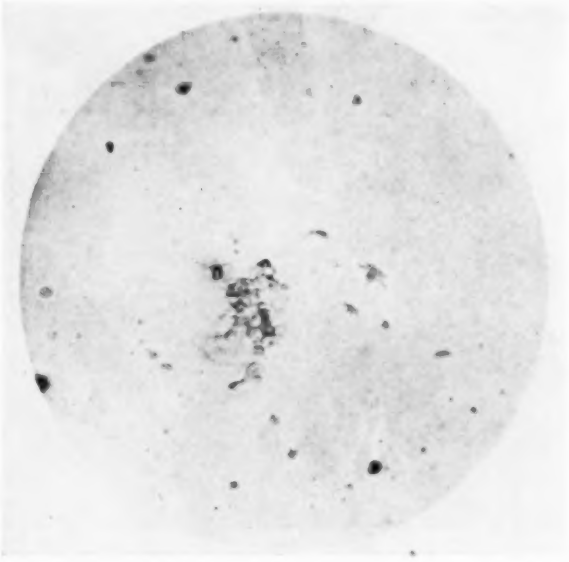

FIG. 1. SOIL THAT HAS BEEN LIMED, BUT WITHOUT OTHER ADDITION, STERILIZED, AND REINOCULATED WITH *Bact. globiforme*, ONE WEEK AFTER INOCULATION

Stained with rose bengal. Magnification 1,000 diameters

organism has a wide choice among carbon and nitrogen sources and can employ carbon compounds as simple in composition as acetic acid.

The method is also being employed here on other soil bacteria and it is felt that it offers considerable promise. It is now being called to the attention of others in the hope that it may be more generally employed. Although it does not offer a means of study-

ing bacteria in unsterilized soil, nor of analyzing the end products of their growth in soil, it does furnish such a satisfactory method of determining their ability to grow in any soil, with or without the addition of nutrients, that it has distinct value in studying the activities of soil bacteria.

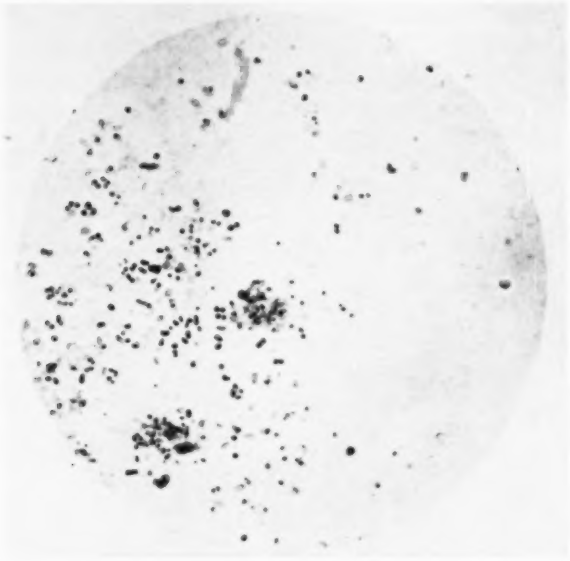

FIG. 2. SOIL THAT HAS BEEN LIMED AND TO WHICH HAS BEEN ADDED GLUCOSE AND AMMONIUM SULFATE, STERILIZED, AND REINOCULATED WITH *Bact. globiforme*, ONE WEEK AFTER INOCULATION

Stained with rosé bengal. Magnification 1,000 diameters. Slightly retouched to improve the definition of the bacteria that were out of focus.

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THE INFLUENCE OF IRON ON THE PIGMENTATION OF ACID-FAST BACTERIA[1]

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Cultures of acid-fast bacteria have generally been observed to vary conspicuously in pigmentation. Colonies of tubercle bacilli growing on either liquid or solid media are generally white or nearly white in the initial stages of growth and usually become yellowish after a few weeks. Frequently the growing pellicle becomes deep yellow, occasionally brown or even reddish, while in other cases no pigmentation is observed at any stage of the growth. Much the same condition has been observed in the case of many of the saprophytic acid-fast organisms especially the smegma, grass and butter bacilli. The presence or absence of pigment in cultures of *M. leprae* has figured conspicuously in the controversial problem of the etiology of leprosy, Duval (1912).

We have used for some time in this laboratory, in the cultivation of both acid-fast and certain non-acid-fast organisms, a medium consisting of fresh infusion of 500 grams of beef, 10 grams peptone, 5 grams Na_2HPO_4, 7 grams glycerol and 15 grams of agar to a liter, autoclaved and adjusted to pH 7.4. For certain purposes this has been supplemented by the addition of 0.2 gram of ferric citrate per liter. Henley (1925) used a similar fluid medium. Analyses of the former, to which no iron was added, show that it contains less than 0.01 mgm. of iron per 100 cc.

All of the organisms mentioned in this paper grow at approximately the same rate on this medium, free of iron or with iron added, as judged by the mass of growth on the surface of agar

[1] Part of an investigation carried out in coöperation with the Canadian National Research Council, Associate Committee on Tuberculosis.

plates or slants. In the case of the acid-fast species tested, there is, however, a great difference in the pigmentation of those grown on the iron-containing and the iron-free media. These results are summarized in table 1. Four cultures of *M. leprae*

TABLE 1

Pigmentation of certain acid-fast bacteria grown on organic media with and without iron

	12 DAYS GROWTH		30 DAYS GROWTH	
	With iron	No iron	With iron	No iron
Human tubercle bacillus:				
Koch	No pigment	No pigment	Brown	No pigment
Upt. A	Creamy	No pigment	Yellow	No pigment
Irv	No pigment	No pigment	Yellow	No pigment
S. N	No pigment	No pigment	Brown	No pigment
Bovine tubercle bacillus:				
599	Deep yellow	No pigment	Deep yellow	No pigment
112	Colorless	No pigment	Yellow	No pigment
B.C.G	Pale creamy	No pigment	Yellow	No pigment
Avian tubercle bacillus:				
10	Pale yellow	No pigment	Yellow brown	Creamy
11	Pale yellow	No pigment	Yellow	No pigment
	4 DAYS GROWTH		10 DAYS GROWTH	
	With iron	No iron	With iron	No iron
M. leprae:				
Clegg I	Deep yellow	No pigment	Deep yellow	Creamy
Clegg II	Deep brown	No pigment	Deep brown	Creamy
65 Duval	Brown	Pale yellow	Deep brown	Pale yellow
68	Brown	No pigment	Deep brown	Creamy
M. phlei	Brown	No pigment	Deep brown	No pigment
M. smegmatis	Yellow	No pigment	Yellow	No pigment

were used, Clegg I and II obtained from the Lister Institute and numbers 65 Duval, and 68 from the American Type Culture Collection with cultures of *M. smegmatis* and *M. phlei* from the former collection. Cultures of these three species showed some

yellow pigment on the iron containing medium almost as soon as the colonies could be distinguished and at the end of four days growth they were deeply pigmented, while at the same time a similar amount of growth on the iron-free medium remained without pigmentation.

The very rapidly growing tubercle bacillus, bovine 599, obtained from the American Type Collection produced a deeply pigmented growth on the surface of the iron-containing medium in six to eight days, while a similar growth on the iron free medium remained non-pigmented. A culture of Calmette's B.C.G. produced a pale yellow growth in ten to twelve days and a deep yellow pigmented growth in twenty to thirty days on the medium with iron while cultures on the iron-free medium exhibited no color. Two recently isolated avian strains were both slightly pigmented on the iron-containing medium after ten days and deeply pigmented after thirty days growth, in contrast with the white or at most cream colored growth on the iron-free medium. Several slower growing cultures of the tubercle bacillus, human Koch strain, from the Lister Institute, three cultures recently isolated from sputum of pulmonary cases and bovine 112 from the American Type collection formed definite colonies by the tenth to twelfth day though they were without color on both media but by the thirtieth day the growth on the iron-containing agar was conspicuously pigmented while similar sized colonies on the iron-free medium remained colorless or white.

In sharp contrast to the behavior of the acid-fast forms all the pigmented non-acid-fast bacteria tested, including several species of the genera, Staphylococcus, Flavobacterium, Pseudomonas and Bacillus produced approximately equal amounts of pigment on both the iron-free and the iron-containing media, table 2.

When the tubercle bacilli of this series were grown on the synthetic medium, described in a previous paper (Reed and Rice, 1928), consisting of asparagin, Na_2HPO_4, NaCl, Na citrate, and ferric sulphate a heavy deep yellow pellicle developed in two to five weeks. On the same medium except for the absence of iron, a similar mass of pellicle, although as previously shown less in weight, exhibited no pigmentation.

The presence of citrate in this medium, as previously shown (Reed and Rice, 1928), stabilises the iron, probably through the formation of complex ions, so that the iron remains in solution up to approximately pH 8.0. In more alkaline solutions, even in the presence of the citrate, iron is precipitated. Corresponding with this, the 599 bovine tubercle bacillus growing on this medium at more acid reactions than pH 8.0 shows definite pigmentation while a more alkaline reaction of the medium results in growth

TABLE 2

Pigmentation of certain non-acid-fast bacteria grown on media with and without iron

	WITH IRON	WITHOUT IRON
Staphylococcus:		
Species 1	Deep yellow	Pale yellow
Species 2	Creamy	Creamy
Species 3	Lemon yellow	Lemon yellow
Species 4	White	White
Species 5	Pink	Pink
Flavobacterium:		
Species 1	Brown	Yellow
Species 2	Yellow	Yellow
Pseudomonas:		
Flourescens	No pigment	Green pigment
Species	Green pigment	Green pigment
Bacillus:		
Species 1	Pale yellow	Pale yellow
Species 2	Pink	Pink

without pigment. This possibly accounts for the fact that Long and Sibert (1926) found only slight increase in growth in their synthetic medium on addition of iron and also that the pellicle was pure white.

CONCLUSIONS

Data have been presented which indicate that the yellow, brown or red pigmentation of acid-fast bacteria is related to the presence of iron in the culture media. Lack of stabilizing substances or high pH which permits precipitation of the iron pre-

vents pigment formation. Non-acid-fast bacteria are not so affected by the iron content of culture media.

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CLASSIFICATION OF THE STREPTOCOCCI OF HUMAN FECES[1]

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The use of fermentative reactions in carbohydrate media for tracing the relationships of the streptococci was first suggested by Gordon in 1904 and elaborated by Houston in 1905 and by Andrewes and Horder in 1908. The last two were able, from their own results and those of Gordon, to divide the streptococci into seven sub-groups or species which they considered main types or type centers about which the more variable forms were clustered. This paper is concerned with their sixth type, *Strep. fecalis*, which ferments sucrose, lactose, salicin, coniferin and mannitol.

In general, the problem resolved itself into the isolation and differentiation of the streptococci found in some thirty specimens of human feces. Over five hundred cultures were isolated and classified according to their fermentative reactions.

The media used were prepared according to standard methods. It was found, however, that the best growth was obtainable at a pH of 7.2 to 7.4 and, furthermore, that when 1 per cent each of glucose and lactose was added to the agar plates a more vigorous growth was produced.

In isolating the streptococci, a portion of a fresh stool was mixed thoroughly with sterile saline solution and one loopful smeared over three agar plates. After a forty-eight-hour growth the colonies of streptococci were located with a hand lens. In order to insure growth it was found necessary to cut the agar bordering the colony and to transfer the whole colony to a glucose sugar broth tube. Although the chance of contamination was

[1] This research was carried out under the direction of Dr. C. A. Stuart, Professor of Bacteriology, Brown University, Providence, R. I.

increased, it was found that by using the hand lens any minute colonies in close proximity to the colony chosen could be detected and avoided. The formation of acid without gas in forty-eight hours was considered a partially confirmed test for streptococci and the test was considered complete with microscopic demonstration of the morphology.

Morphologically, all the strains found were much larger in diameter than the true hemolytic streptococci (i.e., the Beta and Gamma types of the Brown Classification). There are fewer cocci to a chain; the cocci grow much better on agar than the true hemolytic streptococci, but by no means can their growth on agar be called prolific. Only in one strain (VI) was the growth on agar good. In all the other five strains the growth on agar was scanty and veil-like, sometimes difficult to see unless held in direct light. The morphology of the streptococci does not help much in their differentiation. When grown on different media few changes take place. It was found that the chains could be lengthened somewhat by the use of broth to which a small amount of blood serum had been added. It was also found that the diameter of the cocci could be increased by growth on a specially prepared peptic digest medium. On the whole, the morphology was as constant as the fermentative reactions, which did not vary during the time they were under investigation.

PHYSIOLOGY

The streptococci isolated were first differentiated by their fermentative reactions. During the earlier part of the research twelve carbohydrates were used: glucose, lactose, sucrose, salicin, maltose, mannitol, dulcitol, inulin, dextrin, xylose, raffinose and galactose. After two months' work with these twelve carbohydrates it seemed advisable, because of their continued negative results, to drop inulin, xylose, dextrin, raffinose and dulcitol. At no time were the streptococci able to form acid in these five carbohydrates.

During the investigation cultures of the various strains were from time to time saved as stock. Some of these cultures were carried eight months and some more than fifteen months. Every

two weeks the stock cultures were checked in the seven sugars used in order to determine whether they still gave the same fermentation reactions; but in no case did they vary.

Six distinct types were isolated and differentiated according to their fermentative ability. All types do not necessarily appear in a single stool although in the majority of cases such was the case. The types isolated gave the reactions shown in table 1.

Strain I is the *Strep. fecalis* which has been isolated and classified previously. The other five are types that vary definitely from the type of Andrewes and Horder.

A culture of each type has been studied for over fifteen months and each gives the same fermentative reactions that it did when isolated.

TABLE 1*

STRAIN NUMBER	GLUCOSE	LACTOSE	SUCROSE	SALICIN	MALTOSE	GALACTOSE	MANNITOL
I	+	+	+	+	+	+	+
II	+	+	−	+	+	+	+
III	+	+	−	+	+	+	−
V	+	+	+	+	+	+	−
VI	+	+	+	−	+	+	−
VII	+	−	+	+	+	+	+

* In January, 1929, two years after beginning the research, I had the opportunity to recheck the fermentative reactions of the cultures as listed in this table. The reactions were unchanged.

Specimens from cases of jaundice, dysentery and typhoid were used, as well as normal stools, and it is an interesting fact that the various strains appeared in about the same percentage in the abnormal as in the normal stools.

It seemed advisable to determine whether or not any of the strains isolated would produce hemolysis of red cells. Each was accordingly inoculated in blood plates and grown for forty-eight hours. Strains I, III and V produced a small clear zone of hemolysis about the colonies and there was a slight greenish tinge on each of the three plates. This type of reaction is similar to that produced by *Strep. viridans*, and according to the Brown Classification these strains would be classified under the Alpha

type. However, the hemolysis found was not by any means similar to that shown by the true hemolytic streptococcus, being much smaller in area. Strains II, VI and VII produced no hemolysis and no methemoglobin. In order to determine the serological relationship of the strains isolated, six vaccines were made, one from each strain. Over a period of eight weeks increasing amounts of the vaccines were injected into rabbits in five-day intervals. At the end of the period the following titers were obtained:

Strain I, dilution 1–40960
Strain II, dilution 1–160
Strain III, dilution 1–10240
Strain V, dilution 1–40960
Strain VI, dilution 1–160
Strain VII, dilution 1–80

TABLE 2

STRAIN NUMBER	SERA I	SERA III	SERA V	SERA II	SERA VI	SERA VII
I	40960	5120	10240*	10240	640	640
III	10240	10240	5120	10240	1280	640
V	10240	5120	20480	10240	1280	640
II	80	40	20	160	20	40
VII	10	80	10	80	10	40
VI	Negative	Negative	Negative	Negative	160	Negative

* *Strep. fecalis.*

The serological relationship was determined by cross agglutination tests the results of which are given in table 2.

Figure 1 which shows this serological relationship graphically, shows at a glance the rather close relationship of strains I, III and V in comparison to strains II, VI and VII. The figure is plotted on a semi-logarithmic basis in order to include all strains—some having very low dilutions, while others had comparatively high ones.

The cross agglutination tests would indicate a grouping of strains I, III and V and of strains II and VII, whereas strain VI is apparently in a class by itself.

Strains I, III and V, it will be seen, are strong both serologically and antigenically; strains II and VII are weak serologically and fair antigenically, whereas strain VI is negative serologically and only fair antigenically.

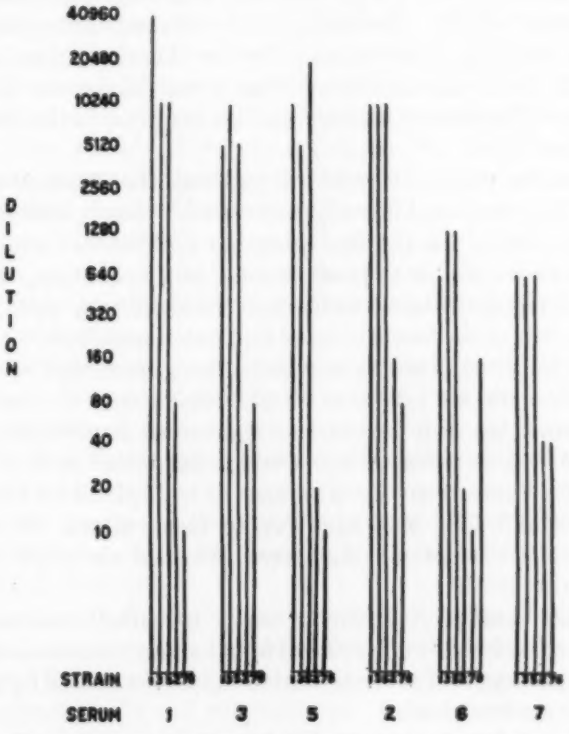

FIG. 1. SEROLOGICAL RELATIONSHIP OF STRAINS 1, 3 AND 5 COMPARED WITH STRAINS 2, 6 AND 7 (SEMI-LOGARITHMIC)

DISCUSSION

In a study of streptococci, not only fecal forms but others, it is extremely difficult to differentiate strains by fermentative reactions. Most workers on the subject have found this true because of the variation in fermentation. Gordon and Houston in 1904, Andrewes and Horder in 1906, and Winslow and Palmer

in 1910, all claim a variation in the strains studied. Many therefore have differentiated the streptococci by the percentage of acid produced in the sugars, glucose being the most popular. Winslow and Palmer concluded from their investigations that the presence of over 3.5 per cent acid in glucose was characteristic of human stools. Secondly, that raffinose-fermenting forms appear to be more abundant in bovine than in human feces, and thirdly, that mannitol-fermenting forms make up about one quarter of human streptococci and are very rare in the feces of the horse and cow.

From the point of view of this paper all the results obtained by these last mentioned investigators coincide closely with the results of this study. In the first place no streptococci were isolated which were not able to produce some acid in glucose. Secondly, no raffinose-fermenting forms were isolated and, thirdly, about 25 per cent of the forms isolated fermented mannitol.

The question arises as to whether such fermentative reactions as were made are fair tests for differentiation. Do the streptococci maintain their fementative characteristics over long periods of time? This investigation would indicate that such is the case. The six strains were carried on agar at a pH of 7.2 for over fifteen months and they still ran true to form, giving the identical fermentative reactions that were obtained on direct isolation from fresh stools.

Philip Hadley of Michigan notes (personal communication) that he and his co-workers have found a variation in *Strep. fecalis* in morphology and in fermentative characters caused by a variety of poor environments.

No work was done in this investigation along these lines. The streptococci were carried on standard agar media. Probably variation can be forced, but if one maintains fixed fermentative characters by the use of standard media, it seems possible to differentiate the strains (at least of the feces) in this way.

P. G. Heineman (1915) states that "The fermentative ability of his two strains was not changed materially by repeated transfers through litmus milk." He further states that these fermentative reactions are of particular interest. Several earlier

papers by the same author deal with the variability in fermentative reactions of several other species of bacteria.

Goodman (1908) succeeded in producing a high acid and a low acid form of diphtheria bacilli from one strain. Winslow and Walker (1909) found acid formation by *B. paratyphosus* relatively stable, but made no attempt at variation of environment. Buchanan and Truax (1910) tested inheritance of acid production of *Strep. lacticus*. They concluded that "impressed variations do not appear to be inheritable." Rettger and Sherrick (1911) conclude from a study of several organisms that bacterial variation, at least of fluctuating type, may be brought about by what may be termed "artificial selection."

E. C. Rosenow (1914) in his paper on streptococcus-pneumococcus transmutations, states that the amount of hemolysis on blood plates was not lost but only decreased by repeated transference on such plates.

These conflicting results indicate that in the field of "Microbic Dissociation" (Hadley, 1927), there is much that awaits further experimentation.

There is little doubt that practically all forms of bacteria can be changed in morphological and fermentative characteristics by the use of a variety of environments including passage through animals, but even so this does not clear up the situation, and even involves it more deeply.

If we are to take Hadley's theory of "Microbic Dissociation" as a criterion, then there is no classification of streptococci, fecal or otherwise. Either we must consider fixed and definite fermentative, morphological, and physiological characteristics, or cast aside the previous classifications and consider only the variety of characteristics obtainable through enforced variation.

The streptococci of the feces as studied in this investigation, without the use of unfavorable environments, were constant in their physiological and morphological characters. It would seem, then, that a classification based on the fermentative ability of the streptococci would be logical.

The *fermentative results* indicate that besides *Strep. fecalis* there are five other species of the streptococci of the feces that differ in their fermentative ability.

According to *serological results* the streptococci of the feces are divided differently. There are apparently three groups. These are considered in this paper as follows: group A strains I, III and V; group B, strains II and VII, and group C, strain VI. The strains of group A are strong both antigenically and serologically, those of group B are weak serologically and fair antigenically, while strain VI is negative serologically and only fair antigenically.

On the basis of blood plates the streptococci are divided into two groups. Strains I, III and V all gave a slight hemolysis, which was not true of strains II, VI and VII.

From these two sections of the investigation, i.e., the physiological and serological, the indication is that there are at least two and possibly three separate and distinct types of the streptococci of the feces.

The strains I, III and V (strain I being *Strep. fecalis*) are all closely related; they show no apparent difference in morphology, close relationship on blood plates and close relationship, serologically, although having varying fermentative reactions.

Strains II and VII are also closely related in morphological and serological characteristics.

Strain VI, however, differs considerably from all other strains. It has a growth on agar that makes it easily recognized among all the rest. It is negative serologically and the general results of the work indicate that strain VI is quite separate from the other five strains.

The primary idea of the investigation was to determine whether or not there were in the human feces other streptococci besides *Strep. fecalis*. The results indicate that such is the case. Whether these forms may be called distinct species is an open question. Certainly strain VI, with its negative serological characteristics and peculiar morphology, is distinct from the other five. But whether there are enough distinctive characteristics of the strains I, II, III, V and VII to separate them into species is more doubtful and needs further work.

SUMMARY

The streptococci of the feces are apparently constant in their fermentative ability.

Differentiations should be made only in media which are most favorable for the growth of the organism.

The results indicate that there are six strains common to human stools. Those fermenting (a) all sugars used, glucose, lactose, sucrose, salicin, maltose, mannitol, galactose; (b) all but sucrose; (c) all but sucrose and mannitol; (d) all but mannitol; (e) all but mannitol and salicin; (f) all but lactose.

Serologically, strains (a), (c) and (d) resemble each other and give slight hemolysis. Strains (b), (e) and (f) give no hemolysis and of these (b) and (f) resemble each other serologically while (e) is distinctly different.

There is little in the morphology of the streptococci of the human feces that assists in their differentiation.

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THE GERMICIDAL ACTION OF HALOGEN DERIVATIVES OF PHENOL AND RESORCINOL AND ITS IMPAIRMENT BY ORGANIC MATTER[1]

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INTRODUCTION

A considerable amount of research work has been published in the chemical, biological and medical literature from which a number of conclusions have been drawn regarding the relationship between chemical constitution and disinfectant action. There are also many papers on record which contain a more or less successful attempt to visualize the mechanism of disinfection and to define it, if possible, chemically or physically. Although the disinfectant action of phenol and its derivatives has been known for some time, the ultimate mode of its action upon the microörganisms remains unknown. An attempt has been made by Loew (1893) to correlate the germicidal action of phenol with its reactivity toward aldehyde groups of the protoplasm. Cooper and collaborators (Cooper, 1913; Cooper and Sanders, 1927; Cooper and Mason, 1928) attribute the disinfectant action of phenol to its capacity of acting as a protein precipitant. The assumption made by Loew cannot hold in all cases because it is known that certain phenol derivatives, such as pentahalogen phenol, are very potent germicides under certain conditions, although by virtue of their constitution they cannot be regarded as capable of a condensation reaction with aldehyde groups. Regarding the work by Cooper and his collaborators, one may

[1] Presented in part before the Division of Biological Chemistry at the Meeting of The American Chemical Society in Swampscott, Massachusetts (September, 1928).

say that the precipitation of proteins by phenol and its derivatives may very well be preceded by a chemical reaction between the disinfectant chemical and the protein; besides, it appears from a number of references in the literature, that disinfectant action in the presence of protein need not be accompanied by the precipitation of the latter.

From the findings of other investigators, hitherto published, it is rather difficult to obtain a definite idea which could be made the basis of a working hypothesis for the subject under consideration. With the experimental evidence on hand it appears to be impossible to say whether a simple penetration of the disinfectant compound into the living cell or a change of the physical-chemical conditions within, or a chemical reaction with definite cell constituents, or some other process, is responsible for what eventually appears to be the "germicidal action." More recently there has been a tendency to attribute the germ killing action to more or less definite chemical reactions, (e.g. formation of salts or salt like compounds), which might be accompanied by other reactions (Schneider, 1926).

An attempt to base the bacteriostatic action of organic dyes upon the assumption of a reaction between definite chemical groups was made by Stearn and Stearn (1926). According to Simon and Wood (1914) there are in the bacterial organism receptors for either acidic or basic substances; this might be represented by a type formula $R\frac{NH_2}{COOH}$ the amino group acting as a receptor for acid substances and the carboxyl group as a receptor for basic ones. A simpler formula would be HPrOH. Simon and Wood assume that the dye enters into a peptide like combination with the cell-protein which according to Stearn and Stearn might in the case of a basic dye be represented by the equilibrium

$$D^+ + PrOH^- \rightleftharpoons DPrOH$$

where D^+ is the cation of the dye DOH, $PrOH^-$ the anion of the receptor HPrOH and DPrOH the un-ionized dye-protein compound.

The participation of a definite chemical group in the bacteri-

cidal action of organic arsenicals was assumed by Voegtlin, Dyer and Leonard (1925). These authors believe that the effectiveness of arsenicals depends upon their reaction with the SH-group; the latter occurs in compounds such as cystein and glutathione, which, as is known from other work, are essential for a number of biological processes taking place in the cell. It is, therefore, clear that the inactivation of such compounds, will lead to serious interference with the life process of the cell and ultimately to its death. The conclusions of Voegtlin and collaborators have been disputed by Schnitzer (1928).

The determination of specific chemical groups of cell substances which play a part in the fixation of the toxic molecule, has not as yet been accomplished directly, but such determination might be attempted by a process of elimination. Thus, if it be found that certain substances inhibit the germicidal action, e.g., of phenol, one could assume that groups which are responsible for the fixation of phenol to this substance and its inactivation in a germicidal sense, are also present in the cell. It is our intention to use this procedure in our attempts to contribute to the elucidation of the mechanism of disinfection by phenol, resorcinol and their halogen derivatives.

From older work, which has direct reference to the germicidal action of polyhalogen phenol derivatives and its reduction by organic matter, the paper by Bechhold and Ehrlich (1906) appears to be the most important. These authors endeavored to find a compound which would be satisfactory for "internal" antisepsis, i.e., would show a pronounced bacteriotropic and at the same time a minimal organotropic action. In the series of the halogen derivatives of phenol, they tested tri-, tetra- and pentahalogen phenol. They conclude from their work that "the introduction of halogen (Cl, Br) into phenol increases its germicidal action with the number of halogen atoms introduced (1 molecule of penta-bromo-phenol having the same effect upon *B. diphtheriae* as 500 molecules of phenol)" and that "the most effective disinfectants (such as tetra-bromo-o-cresol or tetra-chloro-o-biphenol) fail to act in the presence of serum, although they do not precipitate it." These conclusions would lead one to assume that

halogen substitution produces compounds, the germicidal efficacy of which is impaired by organic matter. It was later shown, however, by Laubenheimer (1909) that mono-halogen substituted alkyl derivatives of phenol, although much more powerful than the unsubstituted compounds, are only partly inactivated in the presence of serum.

An important contribution to the problem of inactivation of phenol derivatives by organic matter has been made by Sabalitschka (1928). This author has shown that while the germicidal effect of p-hydroxy-benzoic acid is very considerably reduced in the presence of protein matter, the efficacy of its alkyl esters is practically the same as in the absence of organic matter. He explains this by assuming that the compound with a free carboxyl group, is capable of entering into chemical combination with the organic matter, which does not take place in the case of the ester.

Attention should finally be called to a paper by Klarmann and Von Wowern (1929), who studied the influence of halogenation of 2,4-dihydroxydiphenylmethane upon the germicidal efficacy and its reduction by organic matter. In this case, also, a very considerable increase of germicidal efficacy was observed, different results having been obtained depending on whether the halogen was introduced into the dihydroxy-substituted or the unsubstituted nucleus. The reduction of germicidal efficacy of these compounds by standard[2] organic matter was very pronounced.

EXPERIMENTAL

We assumed that a closer investigation of the disinfectant action of the halogen derivatives of phenol and resorcinol by means of standard methods and of its impairment by organic matter, would simplify the classification of the various subproblems and would lead to a choice of satisfactory chemical and bacteriological testing material for further work.

Standard bacteriological technique

In this investigation, two microörganisms, namely *B. typhosus* and *Staph. pyogenes-aureus*, were used. Fresh twenty-four-hour

[2] As specified by the method of the Hygienic Laboratory (1912).

broth cultures were taken. The composition of broth was as follows: Liebig's beef extract "Lemco" 0.5 per cent, "Difco" peptone 1 per cent and sodium chloride 0.5 per cent, reaction adjusted to pH 6.8. The germicidal efficacy of the various compounds was tested by exposing 0.5 cc. of culture to the action of 5 cc. of the diluted disinfectant for a period of five, ten and fifteen minutes respectively, transferring a loopful into fresh media and incubating for forty-eight hours, after which the final readings were made. In experiments with organic matter, a standard mixture was used consisting of 10 per cent "Difco" peptone and 5 per cent gelatin, 1 cc. of this mixture being added to 5 cc. of

TABLE 1

Phenol; minimum concentrations required for killing B. typhosus and Staph. aureus

	B. TYPHOSUS									STAPH. AUREUS								
	A without organic matter				B with organic matter					A without organic matter				B with organic matter				
	Time of exposure									Time of exposure								
	5 minutes	10 minutes	15 minutes	Average	5 minutes	10 minutes	15 minutes	Average	Ratio $\frac{B}{A}$	5 minutes	10 minutes	15 minutes	Average	5 minutes	10 minutes	15 minutes	Average	Ratio $\frac{B}{A}$
Dilutions (average of 25 experiments):																		
Calculated....	81.6	90.4	96.0	89.3	69.6	75.6	80.4	75.2	0.84	54.4	58.8	61.2	58.1	42.0	50.0	50.0	47.3	0.81
Practical.....	80	90	100	90.0	70	75	80	75.0		55	60	60	58.3	40	50	50	46.6	

the disinfectant prior to the addition of 0.5 cc. of culture, so that in the actual test 2 per cent of peptone and 1 per cent of gelatin were present.

RESULTS

The resistance of the germs used may be seen from table 1, containing the maximum dilutions of phenol at which *B. typhosus* and *Staph. pyogenes-aureus* are killed, both in the absence and presence of organic matter at 20° after five, ten and fifteen minutes. The average figures have been calculated from 25 generally agreeing determinations.

It appears from this table that the presence of organic matter effects a reduction of the germicidal efficacy of phenol against both *B. typhosus* and *Staph. pyogenes-aureus*; the reduction is not very pronounced, the average ratio of the germ killing power in the absence and the presence of organic matter being 0.84 in the case of *B. typhosus* and 0.81 in that of *Staph. pyogenes-aureus*.

In order to ascertain the influence of halogen substitution upon the bactericidal efficacy and its reduction by organic matter, a

TABLE 2

Halogen derivatives of phenol; phenol coefficients

	B. TYPHOSUS				STAPH. AUREUS			
	Without organic matter	With organic matter			Without organic matter	With organic matter		
		Calculated	Corrected	Reduction of efficacy		Calculated	Corrected	Reduction of efficacy
				per cent				*per cent*
o-Chloro-phenol	3.6	3.7	3.1	14	3.8	3.7	3.0	21
p-Chloro-phenol	3.9	3.8	3.2	16	4.0	4.1	3.3	17
m-Chloro-phenol	7.4	6.2	5.2	30	5.8	5.2	4.2	27
2,4-dichloro-phenol	13.3	11.4	9.6	28	12.7	12.2	9.9	22
2,4,6-trichloro-phenol	22.6	15.2	12.8	44	25.0	12.9	10.4	58
2,4,6-trichloro-phenol saturated aqueous solution	21.1							
o-Bromo-phenol	3.8	3.4	2.8	25	3.7	3.1	2.5	32
p-Bromo-phenol	5.4	4.8	4.0	25	4.6	5.0	4.1	12
2,4-dibromo-phenol	18.8	18.0	15.1	20	21.9	17.7	14.3	35
Phenol	1	1	0.84	16	1	1	0.81	19

series of halogen derivatives of phenol was tested, the results of which appear in table 2.[3]

[3] We have found that initial solutions are prepared conveniently by dissolving the chemical in alcohol first and then diluting with water. The final solutions did not, however, contain more than 5 per cent alcohol with exception of the case of tri-chloro-phenol. A small quantity of alcohol does not influence the results to any great extent; of course, appreciable quantities of alcohol may be expected to change the findings markedly since alcohol is known to influence the bactericidal properties of organic compounds in both ways (Koch, 1881).

In the case of tri-chloro-phenol, a larger quantity of alcohol had to be applied because of the unsatisfactory solubility of tri-chloro-phenol in water. Thus

According to this table, the germicidal efficacy increases directly with the number of atoms of halogen introduced. Bromo-substitution leads to compounds which are germicidally more active than the corresponding chloro derivatives. The degree of reduction of bactericidal efficacy by organic matter also depends upon the number of halogen atoms in the compound. While in the columns under the heading "With Organic Matter Calculated" the germicidal efficacy of phenol is regarded as

TABLE 3

Certain alkyl phenols and their halogen derivatives; phenol coefficients

	B. TYPHOSUS				STAPH. AUREUS			
	Without organic matter	With organic matter			Without organic matter	With organic matter		
		Calculated	Corrected	Reduction of efficacy		Calculated	Corrected	Reduction of efficacy
				per cent				*per cent*
m-Cresol	2.5	2.4	2.0	20	2.4	2.4	1.9	19
4-chloro-m-cresol	30.5	26.1	21.9	28	19.5	12.2	9.9	49
Xylenol	5.0	5.0	4.4	12	5.6	4.0	3.2	42
Chloro-xylenol	70.9	60.3	50.6	29	38.7	11.1	9.0	77
Thymol	33.2	32.5	27.3	18	23.6	11.5	9.3	61
4-chloro-thymol	61.3	25.9	21.7	65	158.0	70.7	57.3	64
4-Bromo-thymol	12.5				194.5			
Carvacrol	39.9	30.6	25.7	36	17.8	6.0	4.7	73
4-bromo-carvacrol	25.0				126.7			
Phenol	1	1	0.84	16	1	1	0.81	19

unity, under the column with the heading "Corrected," the figures are based upon the germicidal efficacy of phenol in the absence of

the final dilution in which no growth of *B. typhosus* occurs, viz., 1:2000, contains 9 per cent of alcohol; that in which no growth of *Staph. pyogenes-aureus* occurs, viz., 1:1500, contains 11 per cent of alcohol. Therefore, the figures in the fifth horizontal column of table 2 do not represent entirely objective values; on the other hand it is not probable that the alcohol plays an important rôle even in this case, because a saturated solution of tri-chloro-phenol in water, which corresponds very nearly to a dilution of 1:2000, gives a phenol coefficient of 21.1 (with horizontal column) based upon the dry content, in satisfactory agreement with the figure of 22.6 obtained with a solution containing 9 per cent alcohol.

organic matter being 1. m-Chloro-phenol seems to be an exception, in that it is germicidally more active, and the reduction of its germicidal efficacy by organic matter is more pronounced, than in the case of its isomers.

There is also a direct relationship between the degree of reduction of the germicidal power by organic matter and the number of halogen atoms in the phenol derivative.

Table 3 illustrates the influence of halogenation of certain alkyl derivatives of phenol upon the germicidal action. In all cases halogenation produces a very marked increase of germicidal efficacy. Considerable impairment of the disinfectant action is observed in the case of certain alkyl derivatives of phenol, the difference between the efficacy in the absence and in the presence of organic matter being particularly noticeable in tests with *Staph. pyogenes-aureus*. Thus m-cresol and xylenol show about the same germicidal efficacy against *B. typhosus* and *Staph. pyogenes-aureus* and there is no pronounced impairment of the germicidal efficacy by organic matter. On the other hand, organic matter reduces the disinfectant action of thymol and carvacrol against *Staph. pyogenes-aureus* more than 50 per cent, while the reduction in the case of *B. typhosus* is comparatively insignificant.

The germicidal efficacy of 4-chloro-m-cresol is considerably greater than that of the unsubstituted m-cresol, the increase being much more pronounced against *B. typhosus* than *Staph. pyogenes-aureus*. The impairment of germicidal action by organic matter is somewhat more pronounced in the case of *Staph. pyogenes-aureus*. Still higher figures are obtained with chloroxylenol, the germicidal efficacy of which is very markedly reduced by organic matter in the case of *Staph. pyogenes-aureus*. With chlorothymol one has the strange phenomenon of the germicidal efficacy against *Staph. pyogenes-aureus* being much more pronounced than against *B. typhosus*. With bromothymol and bromocarvacrol similarly high figures against *Staph. pyogenes-aureus* are obtained in the absence or organic matter. However, extremely varying results appear in other cases; it is impossible

to account for these variations at the present time (they were, therefore, left out in the table).[4]

The series of halogen derivatives of resorcinol was investigated in a similar manner. Although the introduction of alkyl groups into the nucleus of phenol is known to lead to derivatives showing great germicidal potency [Simon and Wood (1914)], one finds that halogenation of resorcinol, although leading to compounds which are much stronger disinfectants than resorcinol itself, does not produce derivatives of unusually high germicidal action, comparable, e.g., to that of the alkyl derivatives of resorcinol; this is clearly in contrast to the corresponding conditions in the case of phenol.

TABLE 4

Resorcinol and its chloro and bromo derivatives; phenol coefficients

	B. TYPHOSUS				STAPH. AUREUS			
	Without organic matter	With organic matter			Without organic matter	With organic matter		
		Calculated	Corrected	Reduction of efficacy		Calculated	Corrected	Reduction of efficacy
				per cent				*per cent*
Resorcinol	0.3	0.3	0.25	17	0.4	0.3	0.24	39
4-chloro-resorcinol	0.7	0.5	0.42	40	1.0	0.6	0.48	52
4,6-dichloro-resorcinol	3.2	2.0	1.7	47	3.9	2.2	1.8	54
2,4,6-trichloro-resorcinol	5.0	2.3	1.9	61	4.3	2.4	1.9	55
4-bromo-resorcinol	1.0	1.0	0.84	16	1.25	0.8	0.65	48
4-6-dibromo-resorcinol	4.0	2.7	2.3	43	4.5	2.0	1.6	64
2,4,6-tribromo-resorcinol	6.7	2.9	2.4	64	6.4	2.7	2.2	66

Table 4 illustrates this. Mono substitution of resorcinol leads to compounds which are 2 to 3 times more germicidal than resorcinol but not stronger than phenol. The di-substituted compounds are 11 to 13 times, and the tri-substituted 16 to 22 times more germicidal than resorcinol. As in the case of phenol, bromo

[4] Most compounds enumerated in table 3, and particularly the halogen derivatives, are extremely little soluble in water. They were, therefore, used in soap emulsions for the bacteriological test. In view of the well known fact that the quantitative relationship between the amount of soap and that of the disinfectant compound sometimes influences the germicidal efficacy of the latter, the figures in table 3 are of relative importance.

substitution leads to compounds which are more germicidal than those obtained by substitution with chlorine. Organic matter effects a reduction of the germicidal efficacy on the average of more than 50 per cent in the case of the di- and tri-substituted halogen derivatives of resorcinol.

Influence of the media upon the bactericidal action

The considerable reduction of disinfectant action by organic matter previously dealt with suggested the possibility that the small quantity of organic matter present in the media might affect the disinfectant action to some extent. In other words, it was reasonable to suppose that if the culture were entirely freed from its media, it would be more accessible to the action of the disinfectant than when brought in contact with the disinfectant solution with the media adhering. In order to determine whether this is the case, tri-chloro-phenol was tested first, because in the case of this substance, the most distinct reduction of germicidal efficacy by organic matter was expected. When the experiment corroborated our surmise with reference to this chemical, the investigation was extended to dichlororesorcinol. This substance was chosen because it combines the advantage of satisfactory solubility in water with a sufficient sensitiveness of germicidal efficacy to the presence of organic matter. It was also necessary to make sure that the viability of the germ was not markedly impaired by the process of centrifuging. This was done by testing the resistance of the germ against phenol under the same conditions.

The following procedure was adopted: 10 cc. of culture was first centrifuged and the clear supernatant liquid drawn off. Then the bacterial residue was stirred up with a sterile platinum wire and 10 cc. of sterile physiological salt solution added. After thorough mixing the tube was centrifuged. The supernatant liquid was drawn off and the residue stirred with a fresh portion of physiological salt solution and filtered. Five-tenths cubic centimeter of the suspension was removed and mixed with the diluted disinfectant. It may be assumed that by this method practically all organic matter is removed.

TABLE 5

Phenol, trichloro-phenol, dichloro-resorcinol; a comparison of the dilutions required for killing "washed" and "not washed" B. typhosus and Staph. aureus

	B. TYPHOSUS									STAPH. AUREUS								
	Without organic matter				Ratio B/A	With organic matter				Ratio B/A	Without organic matter				Ratio B/A	With organic matter				Ratio B/A
	Time of exposure					Time of exposure					Time of exposure					Time of exposure				
	5 minutes	10 minutes	15 minutes	Average		5 minutes	10 minutes	15 minutes	Average		5 minutes	10 minutes	15 minutes	Average		5 minutes	10 minutes	15 minutes	Average	
Phenol:																				
A regular test....	80	90	100	90.0		70	75	80	75.0		55	60	60	58.3		45	50	50	48.3	
B "washed"					1.22					1.02					1.20					1.14
bacteria.....	100	110	120	110		70	80	80	76.6		60	70	80	70.0		50	55	60	55 0	
2,4,6-trichloro-phenol in the presence of alcohol:																				
A regular test....	2,000	2,250	2,500	2,250		1,000	1,250	1,250	1,166		1,250	1,500	1,500	1,417		600	700	700	666	
B "washed"					2.37					1.16					2.23					1.10
bacteria.....	5,000	5,000	6,000	5,333		1,250	1,250	1,500	1,333		3,000	3,000	3,500	3,166		600	800	800	733	
2,4,6-trichloro-phenol:																				
A regular test....		2,000	2,000																	
B "washed"																				
bacteria.....	4,000	4,500	5,000								2,500	2,750	2,750							
4,6-dichloro-resorcinol:																				
A regular test....	250	300	300	283		100	175	200	158		175	250	250	225		80	100	125	102	
B "washed"					2.47					1.00					1.48					1.23
bacteria.....	550	750	800	700		100	175	200	158		250	350	400	333		80	140	160	126	

Table 5 shows the maximum dilutions of the substances tested which kill *B. typhosus* and *Staph. pyogenes-aureus* previously washed with a physiological salt solution. These dilutions are compared with the practical dilutions of the regular tests. The ratio columns indicate the relationship between the dilutions required to kill "washed" and "not washed" bacteria.

It appears first that the presence of the minute quantity or organic matter in the media influences the germicidal action of phenol only slightly. If the germicidal efficacy of phenol against *B. typhosus* and *Staph. pyogenes-aureus* is regarded as 1 in the regular test, then the phenol seems to be 1.22 times more effective against *B. typhosus* and 1.20 times more effective against *Staph. pyogenes-aureus* in the absence of media. Considerably greater differences are observed in the cases of the other two substances. Thus tri-chloro-phenol seems to show more than twice the efficacy against both *B. typhosus* and *Staph. pyogenes-aureus* when the minute quantity of the organic matter from the media is removed. Similar conditions are encountered in the case of di-chloro-resorcinol.

Referring to tri-chloro-phenol, the same remarks hold as given before, viz., the tests in the second horizontal column were carried out in the presence of alcohol, which fact may detract from the objective value of our findings. However, it appears from the tests in the third horizontal column, which refer to tests carried out with a saturated aqueous solution of tri-chloro-phenol, that the difference between tests carried out in the presence and those in the absence of alcohol, cannot be regarded as important. The blank spaces in the table indicate that in these particular cases, the saturated aqueous solution of tri-chloro-phenol (approximately 1:2000) was not sufficiently strong to effect a killing of the germs.

It is also of interest to consider the experiments which were carried out using "washed" bacteria with the subsequent addition of standard organic matter, as required by the specifications of the Hygienic Laboratory method. It appears that in practically all cases the dilution of disinfectant required to kill the "washed" germs in the presence of standard organic matter corresponds

very closely to the concentration required to kill "untreated" germs such as are used in the regular working procedure. From this two conclusions can be drawn:

1. Centrifuging did not impair the viability of the germ because under the conditions of the regular test, the "washed" germ showed the same resistance against the disinfectants tested as the untreated germ.[5]

2. The fact that the germicidal efficacy against "washed" germs is greater is owing practically entirely to the removal of the minute quantity of organic matter, normally present in 0.5 cc. of the culture which is brought in contact with the diluted disinfectant.

Finally another experiment was carried out in order to prove that the small quantity of organic matter present in the media is responsible for the considerable reduction of germicidal efficacy of certain compounds. A comparison of the following two cases suggested itself: In one case the quantity of media which is normally present in the amount of culture used is first brought into contact with the dilute disinfectant and subsequently with a culture previously freed from its media. In the other case the microörganism, freed from its media, is brought into contact with the dilute disinfectant first, and the media added subsequently. If the small quantity of organic matter present in the media actually interferes with the germicidal action of the disinfectant, then in the first experiment the effect would have to be less than in the second; it is understood, of course, that the microörganisms are exposed to the action of the disinfectant for the same period of time in both cases.

The process used is as follows: The culture is freed from its media by centrifuging and treating with a physiological salt solution as described before. In one case ("A") 0.5 cc. of media is added to 5 cc. of diluted disinfectant and allowed to stand three minutes, whereupon 0.5 cc. of "washed" culture is introduced. From this time on the procedure corresponds to that of our regu-

[5] According to Winslow and Brooke (1927), the viability of certain germs is impaired when suspended in distilled water while others are much less sensitive to this treatment.

lar standard bacteriological technique, i.e., one loopful is removed from this mixture and transplanted into fresh media after five, ten and fifteen minutes of contact respectively. In the other case ("B") 0.5 cc. of a suspension of culture in physiological salt solution is added to 5 cc. of disinfectant. After three minutes 0.5 cc. of media is introduced and two minutes later the first transplant is made in fresh broth; the following transplants are made five and ten minutes after the first transplant. Thus, also in this case, the bacteria were in contact with the disinfectant for five, ten and fifteen minutes respectively. In other words, the purpose of this experiment was to obtain a temporal separation of the two factors, namely germ and media, and to subject

TABLE 6

	B. TYPHOSUS					STAPH. AUREUS				
	Time of exposure				Increase of efficacy	Time of exposure				Increase of efficacy
	5 minutes	10 minutes	15 minutes	Average		5 minutes	10 minutes	15 minutes	Average	
					per cent					*per cent*
Phenol:										
A	80	90	100	90	22	50	60	70	60	17
B	100	110	120	110		65	70	75	70	
4,6-dichloro-resorcinol										
A	325	375	400	366	64	175	200	250	208	20
B	550	600	650	600		225	250	275	250	

them to the influence of the disinfectant solution at different times and in different sequences instead of simultaneously.

The experiments were carried out with both *B. typhosus* and *Staph. pyogenes-aureus* using both phenol and dichlororesorcinol as germicides. Table 6 illustrates the conditions encountered.

It appears that there is a very considerable increase in the efficiency of dichlororesorcinol against *B. typhosus* when the operations are carried out according to scheme described under "B." The difference observed in the case of *Staph. progenes-aureus* is much less pronounced. In the case of phenol, no great difference between the two procedures could be expected on the basis of our previous experiments and none was found.

NOTES ON THE PREPARATION OF THE CHEMICAL COMPOUNDS USED IN THIS WORK

The substances used in this work were either prepared in our laboratory or purchased from the Eastman Company. All substances bought were carefully examined for purity, either by a determination of the melting point or by a halogen determination or both.

The determination of halogen was carried out in all cases according to Pregl's micro-modification of Carius' method. In those cases where the purity was not satisfactory for our purposes a further purification was carried out by redistillation or recrystallization.

Following are notes on the preparation of those chemicals which were made in our laboratory.

Preparation of 4-chloro-thymol $C_6H_2(OH)\cdot CH_3\cdot C_3H_7\cdot Cl$

Thymol was dissolved in two parts of carbon tetrachloride. The theoretical quantity of sulfuryl chloride was added drop by drop, with stirring and cooling. The carbon tetrachloride solution was shaken with a 10 per cent sodium hydroxide solution which dissolved the chlorothymol. The alkaline solution was separated from the carbon tetrachloride, washed with ether in order to remove traces of carbon tetrachloride and acidified. The precipitated oily chlorination product was washed with water, dried with sodium sulphate and distilled in a vacuum (5 mm.). The fraction boiling at 110 to 120° solidified. It was remelted and stirred with petroleum benzene until cold. Melting point (uncorrected) 60°, (lit. 59–60°).

Analysis: Substance, 13.284 mgm.: AgCl, 9.840 mgm. Calculated for $C_{10}H_{13}OCl$: Cl, 19.21. Found: Cl, 18.32.

Preparation of 4-bromo-thymol $C_6H_2(OH)\cdot CH_3\cdot C_3H_7\cdot Br$

Thymol was dissolved in two parts of glacial acetic acid. The theoretical quantity of bromine, dissolved in an equal part of glacial acetic acid, was added drop by drop with stirring and cooling. The acetic acid solution was poured into a large quantity of water. The precipitated bromination product was washed

with a 5 per cent solution of sodium carbonate and with water, dried over sodium sulphate and distilled in a vacuum. The fraction boiling between 120 to 135° solidified. It was remelted and stirred with petroleum benzene until cold. Melting point (uncorrected) 56°, (lit. 54–56°).

Analysis: Substance, 9.615 mgm.: AgBr, 7.962 mgm. Calculated for $C_{10}H_{13}OBr$: Br, 34.88. Found: Br, 35.25.

Preparation of 4-bromo-carvacrol $C_6H_2(OH)\cdot CH_3\cdot C_3H_7\cdot Br$

Carvacrol was dissolved in an equal volume of glacial acetic acid. The theoretical quantity of bromine, dissolved in one part of glacial acetic acid, was added drop by drop with stirring and cooling. The acetic acid solution was poured into a large quantity of water. The precipitated crude product was washed with a 5 per cent sodium carbonate solution and with water, dried over sodium sulphate and distilled in a vacuum. The vacuum distillation was repeated and the fraction boiling between 112 to 114° (5 mm.) was isolated.

Analysis: Substance, 11.989 mgm.: AgBr, 9.856 mgm. Calculated for $C_{10}H_{13}OBr$: Br, 34.88. Found: Br, 34.97.

Preparation of 4-chloro-resorcinol $C_6H_3(OH)_2Cl$

This compound was prepared according to Reinhard's (1878) method except for the purification, which was done by recrystallization from carbon tetrachloride instead of by sublimation. Melting point (uncorrected) 89.5°, (Reinhard 89°).

Analysis: Substance, 8.973 mgm.: AgCl, 8.812 mgm. Calculated for $C_6H_5O_2Cl$: Cl, 24.53. Found: Cl, 24.30.

Preparation of 4,6-dichloro-resorcinol $C_6H_2(OH)_2Cl_2$

This compound was prepared by treating a solution of resorcinol in ether with a 6 per cent solution of chlorine in carbon tetrachloride, using an excess of 5 per cent of chlorine over the amount of resorcinol. After the formation of hydrogen chloride fumes had subsided an air current was passed through the mixture in order to remove most of the solvent and of the hydrogen chloride. The remaining concentrated solution was decolorized

by boiling with Darco and filtered. The crude product which crystallized from the filtrate was purified either by vacuum sublimation or by recrystallization from benzene. Melting point (uncorrected) 109°. Yield 75 per cent.

The attempt to prepare this compound according to the method of Reinhard (1878) was unsuccessful. No consistent results could be obtained. The melting point observed by Reinhard was 77°.

Analysis: Substance, 9.965 mgm.: AgCl, 16.162 mgm. Calculated for $C_6H_4O_2Cl_2$: Cl, 39.62. Found: Cl. 40.12.

Preparation of 2,4,6-trichloro-resorcinol $C_6H(OH)_2Cl_3$

This preparation was made according to the method of Benedikt (1884). The substance was purified by recrystallization from boiling water and subsequently from carbon tetrachloride. Melting point 73.5° (uncorrected) in contrast to 83° as reported by Benedikt.

Analysis: Substance, 7.599 mgm.: AgCl, 15.349 mgm. Calculated for $C_6H_3O_2Cl_3$: Cl, 49.84. Found: Cl, 50.00.

Preparation of 4-bromo-resorcinol $C_6H_3(OH)_2Br$

This compound was prepared from 5-bromo-resorcylic acid $C_6H_2(OH)_2 \cdot COOH \cdot Br$ according to the method by Zehenter (1888, a). The bromo resorcylic acid was prepared by bromination of β-resorcylic acid according to Zehenter's method (1888, b). The bromo resorcinol was purified by recrystallization from carbon tetrachloride. Melting point 102° (uncorrected) (Zehenter reports 91°).

Analysis: Substance, 6.620 mgm.: AgBr, 6.522 mgm. Calculated for $C_6H_5O_2Br$: Br. 42.28. Found: Br, 41.92.

Preparation of 4,6-dibromo-resorcinol $C_6H_2(OH)_2Br_2$

This substance was prepared according to the method by Zehenter (1888, c). The product was recrystallized from carbon tetrachloride and from water. Melting point 115.5° (uncorrected) (Zehenter reports 110–112°).

Analysis: Substance, 5.801 mgm.: AgBr, 8.176 mgm. Calculated for $C_6H_4O_2Br_2$: Br, 59.66. Found: Br, 59.98.

Preparation of 2,4,6-tribromo-resorcinol $C_6H(OH)_2Br_3$

In the preparation of this substance the method described by Benedikt (1884) was followed rather closely.

The tribromoresorcinol was purified by recrystallization from water and from carbon tetrachloride. Melting point (uncorrected) 112° (Benedikt reports 111°).

Analysis of substance, 6.689 mgm.: AgBr, 10.899 mgm. Calculated for $C_6H_3O_2Br_3$: Br, 69.13. Found: Br, 69.34.

REMARKS

It appears from our experiments that there exists not only a relationship between the chemical constitution and germicidal action of certain groups of phenol and resorcinol derivatives, but that a definite quantitative relation is also to be found between the chemical constitution of these compounds and the impairment of their germicidal efficacy by organic matter. It is noteworthy that a large quantity of organic matter is by no means necessary to demonstrate this impairment; even the exceedingly small quantity of organic matter present in the media in which the culture is grown suffices to produce considerable differences in the germicidal efficacy of certain compounds.

Although the experimental results of this paper do not as yet permit definite conclusions to be drawn regarding the processes which are responsible for this impairment of germicidal efficacy, the following will give an idea of the working hypothesis on the basis of which we expect to proceed in further work on this problem.

The concept of organic matter, as used in bacteriological phraseology, is rather indefinite from a chemical viewpoint. Both constituents of the so-called organic matter, namely, gelatin and peptone, are products of partial hydrolysis of protein and might, in the light of older and recent studies on the chemical constitution of proteins and their cleavage products, be regarded probably as mixtures of polypeptides, amino-acid anhydrides and amino acids. In any case, the composition of organic matter will differ qualitatively and quantitatively, depending upon the initial material used and the method and duration of protein

hydrolysis applied in the preparation of these constituents of organic matter.

It is assumed that the impairment of the germicidal action of the compounds studied in this paper will be influenced by the presence of certain free reactive groups in these more complex protein cleavage products, such as the NH_2, COOH, OH, NH and SH groups; however, in contrast to the ideas of investigators whose work has been mentioned in the introduction, we believe that such impairment will not be brought about by a definite chemical reaction between the disinfecting compound and the "reactive" group, but rather by the formation of "additive" compounds in which, as in Werner's compounds, the secondary valencies will play an important rôle. Assuming for instance that the reactive group in the protein is the amino group, then the additive compound may be represented thus:

$$\text{Protein—}NH_2\ldots HO \cdot R$$

The degree of impairment of the disinfectant action of a phenol derivative by organic matter will depend upon the stability of the additive compound of the respective phenol derivative and the protein cleavage product bearing the reactive amino group. In this particular instance the stability will depend upon the affinity of the hydroxyl group or groups for the amino group or groups. One factor, which will influence the affinity, will be the "acidity" of the hydroxyl group, which depends, e.g., upon the number of halogen atoms in the nucleus of the phenol derivative and the "basicity" of the amino group which depends, e.g., upon the amino acid or peptide which carries the amino group.

It is obvious that these ideas regarding the impairment of disinfectant action by reactive groups may be applied to the disinfecting action proper, since possibly groups in proteins which hinder disinfecting action, may bear resemblance to those which attach the disinfectant molecule to the cell.

Of course, this does not mean that the importance of other factors, such as distribution coefficient, solubility, surface tension, etc., is disregarded. They probably relate to the physical phases of the process of disinfection which precede the chemical phase,

viz., the fixation of the hydroxyl group of the phenol or its derivative by a reactive group of the protoplasm by means of secondary valencies; such fixation may subsequently produce other changes, e.g., protein precipitation which is regarded by some as the true cause of disinfection by this class of compounds.

SUMMARY

1. The results of experiments upon the disinfectant action of phenol, resorcinol and their halogen derivatives, against *B. typhosus* and *Staph. pyogenes-aureus*, tend to indicate that there is a distinct relationship between the constitution of these derivatives and the impairment of their disinfectant action by organic matter.

2. It is shown that even the exceedingly small quantity of organic matter present in the original culture suffices to produce very considerable impairment of the germicidal efficacy of certain compounds.

3. A working hypothesis of disinfectant action is outlined, depending upon the assumption of formation of so called molecular (additive) compounds of the disinfectant agent with certain free reactive groups in the organic matter, or the protoplasm, respectively, which is to form the basis for further work on the problem.

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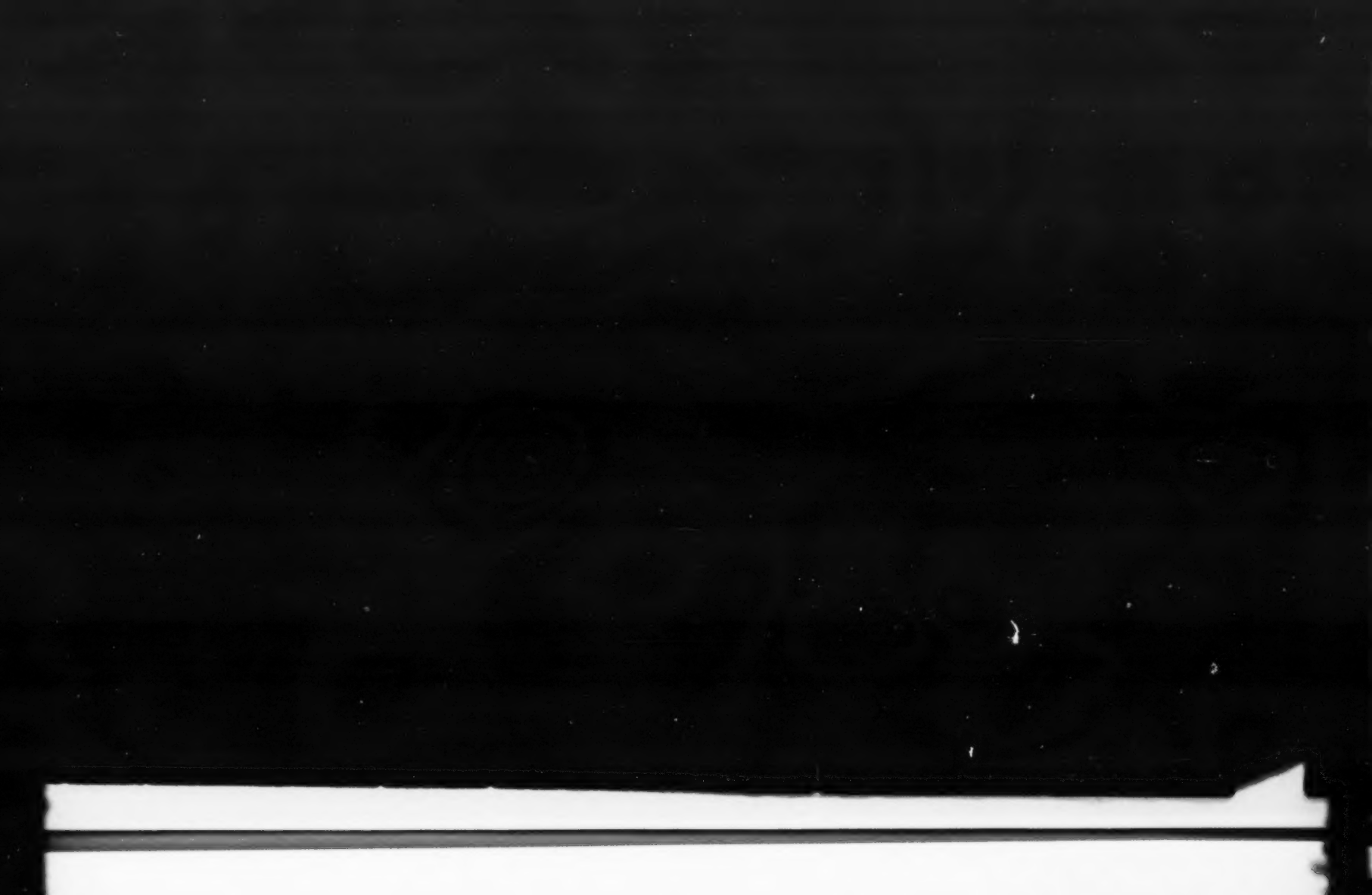

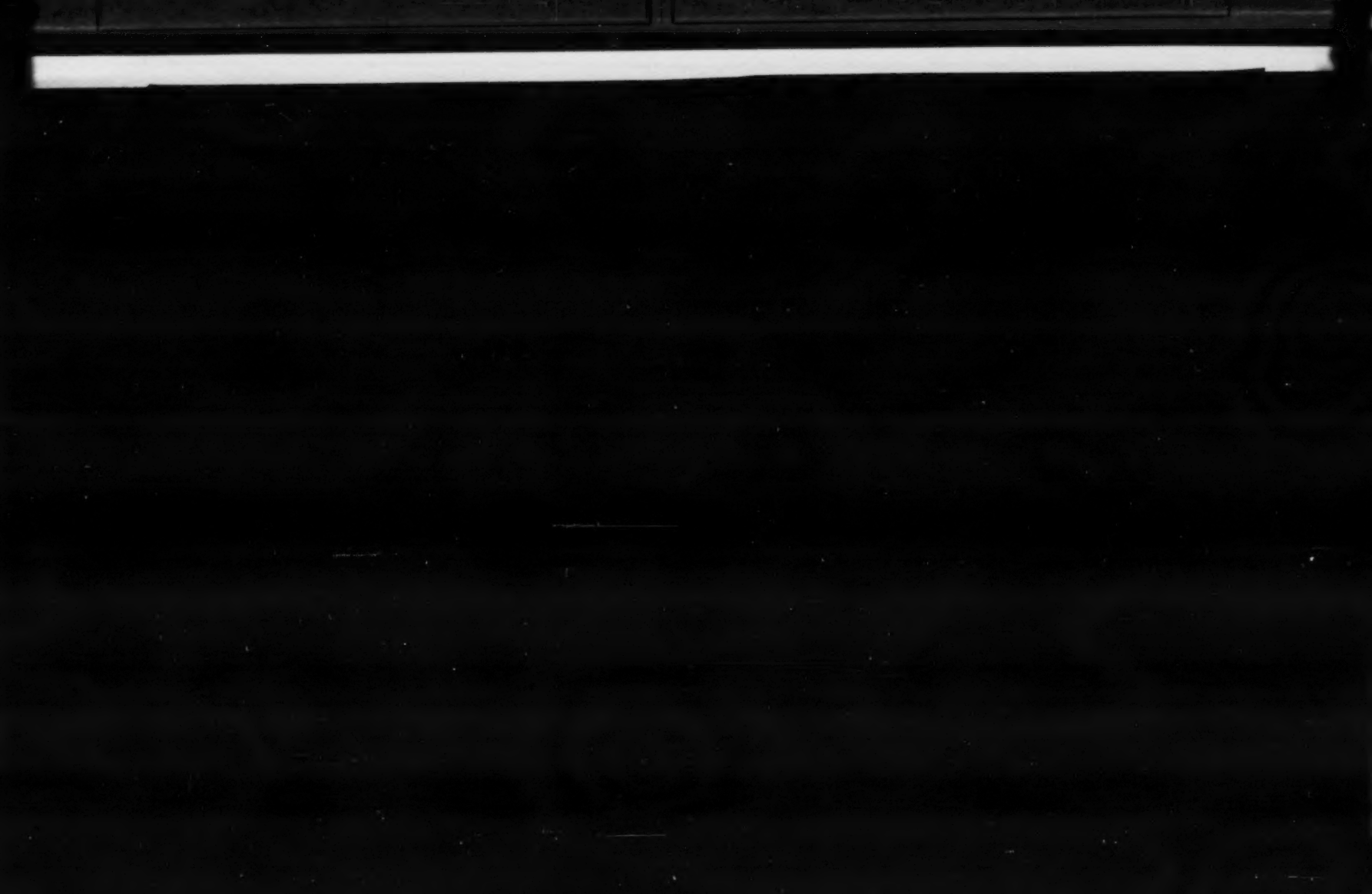

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